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The In Vivo Removal of Cartilage Matrix by Papain

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Cartilage is the simplest connective tissue in the body. More is known of its chemical composition than of any other tissue in the body. Experimental production of *in vivo* changes in cartilage offers a model for a study of the precise ways in which ground substance can be damaged. The widespread *in vivo* removal of cartilage matrix by papain is a change which can be defined on the chemical level.

The intravenous injection of a solution of crude papain into young rabbits resulted within several hours in rapidly progressive loss of rigidity and collapse of the ears. During the following several days, the ears gradually regained their tone and became erect again. The rabbits showed no other ill effects[1].

Histologic examination showed complete loss of basophilia of cartilage matrix in all locations examined which included ear, trachea, bronchi, joints and epiphyses. There was some diminution in the metachromasia of cartilage. During the first several hours, basophilic, metachromatic material accumulated in the connective tissue and lymphatic channels adjacent to cartilage. These findings indicate a liberation of chondroitin sulfate in some form from cartilage resulting in its diffusion into adjacent tissue and removal by lymphatic channels. Recovery of cartilage, as judged histologically, was complete in about two weeks.

There were no histologic changes in tissues other than cartilage. Special study was made of tissues known to contain chondroitin sulfate or other sulfated mucopolysaccharides, such as cornea, heart valves, aorta and skin. No changes in the basophilic or metachromatic staining properties occurred in these tissues.

Chemical analysis of cartilage was performed in this laboratory by Dr. Theodore Tsaltas. He found that 24 hours following papain injection, there was a reduction in the chondroitin sulfate content of cartilage of about 40 per cent. There was an increase in the molar ratio of nitrogen to hexosamine in cartilage, also[2].

In order to study the rate and extent of removal of chondroitin sulfate further, rabbits were given S^{35} several days prior to the injection of papain. It has been shown that most of the S^{35} which is retained in an animal following administration in the form of sodium sulfate is incorporated in the form of sulfated mucopolysaccharides, especially chondroitin sulfate[3]. Rabbits were given papain 6 and 11 days after receiving S^{35}. Autoradiograms showed a reduction in the S^{35} content of cartilage of papain-injected rabbits, compared with control rabbits. Direct counts of radioactivity of cartilage from papain-injected rabbits determined by Dr. Tsaltas showed approximately 40 per cent less activity than control cartilage. He also

found a rapid rise in the blood and urine levels of S^{35} following papain injection.

Intravenous injections of crude papain into mice, chicks, and hamsters resulted in disappearance of cartilage basophilia. Injection of the plant enzymes bromelin and ficin produces a similar change in mice. Further studies on these enzymes are planned.

Occlusion of the arterial supply of one ear for 15 minutes at the time of injection resulted in protection of that ear against collapse. Progressive loss of rigidity and collapse occurred in isolated rabbit ears excised shortly after the intravenous (femoral vein) injection of crude papain. The rate of collapse in such ears showed a marked temperature dependence. Histologic examination showed changes in cartilage similar to those seen *in vivo*. These results indicate that the active factor is rapidly concentrated in cartilage and that the process is enzymatic in nature.

The daily systemic administration of cortisone, hydrocortisone or prednisilone prevented or greatly delayed recovery of cartilage, as shown by the persistence of ear collapse and the continued histologic abnormalities in cartilage. Prolonged cortisone administration in young rabbits following a single injection of crude papain resulted in virtually complete arrest of enchondral bone formation. These results indicate that cortisone and related steroids interfere with the synthesis or deposition of chondroitin sulfate in cartilage.

In an attempt to find the component in crude papain responsible for the effect on cartilage, two crystalline enzymes prepared from papaya latex[4-6] were tested and the following results were obtained. Crystalline papaya lysozyme was without effect on cartilage. Cysteine activated crystalline papain proteinase in relatively large doses (5 to 10 mg.) produced very slight loss of basophilia of cartilage and did not cause ear collapse. However, when crystalline papain was treated with iodoacetamide or p-chloromercuribenzoate prior to injection, amounts as small as 2 mg. produced total ear collapse and complete loss of cartilage basophilia. It is suggested that these sulfhydryl-blocking agents prevent denaturation of the enzyme in the blood, permitting it to reach cartilage where it is reactivated. This hypothesis is strengthened by observations on factors influencing the rate of collapse in isolated rabbit ears removed shortly after injection into the femoral vein. The skin and subcutaneous tissue of the ears were stripped, exposing the cartilage. Following injection of iodoacetamide inactivated crystalline papain, ears placed in phosphate buffer at pH 7 proceeded to collapse, whereas contralateral ears placed in buffer containing p-chloromercuribenzoate or iodoacetamide failed to collapse. On the other hand, placing ears in buffer containing cysteine and versene greatly increased the rate of collapse. These results indicate that the enzymatic activity in isolated ears depends on SH groups and is consistent with the hypothesis that crystalline papain proteinase is the factor responsible for the effect on cartilage. Following intravenous injection of activated crystalline papain, collapse does not occur in isolated ears. This is consistent with the idea that activated crystalline papain fails to reach cartilage.

These results indicate that crystalline papain is the component in crude papain responsible for the effect on cartilage. Further work on this aspect of the problem is in progress.

The chemical site of action of crystalline papain in cartilage has not been determined, but a consideration of the histologic and chemical findings and known enzymatic action of crystalline papain indicates that it probably attacks the protein moiety of the mucoprotein of cartilage, thus liberating chondroitin sulfate. *In vitro* studies of the effect of crystalline papain on cartilage and the mucoprotein of cartilage are in progress and should clarify this point.

Observations on the Structure and Metabolism of Epiphysial Cartilage

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Tonight I should like to present some observations on the structure of epiphysial cartilage, together with certain changes which may be produced by a number of procedures. In the past the upper epiphysis of the growing rat has been studied extensively, and has provided a great deal of information, for instance in experiments dealing with dietary restrictions of various types, including rickets, endocrine disturbances, etc. The growth activity of this tissue is obviously great, since the entire epiphysial plate may be replaced in two to three days' time.

When fresh stained or unstained cartilage cells are observed, one is impressed by their irregular outlines, a point which was mentioned by Virchow over 100 years ago. When fresh preparations are stained with dilute toluidine blue, the cells take the stain while the matrix generally does not. The response of fixed tissue is different. Cells may be freed from their matrix by treatment with trypsin. Such isolated, yet intact, cells can then be fixed and stained on a slide as one would treat a blood smear. The cytoplasmic detail of such cells is well shown.

Further detail is obtained when one observes thin sections (0.5 micron) of cartilage with the light microscope. Here again the irregular boundaries of the cytoplasm are well shown. Finally, when ultra-thin sections are studied with the electron microscope, intracellular detail and the relation between cytoplasm and matrix are strikingly brought out. These observations have been made in collaboration with Mr. A. J. Tousimis. Briefly, the cell has a prominent endoplasmic reticulum consisting of parallel membranes with dense osmophilic granules attached to them. Sometimes the membranes open out into lake-like vesicular structures. So far, only a few poorly defined mitochondria have been seen. The cytoplasm of the cells extends out into the matrix as finger-like projections, so that the entire cell has a sort of a porcupine appearance. The matrix itself consists of fine unbanded fibers all having approximately the same diameters.

One can then go on to obtain a more precise picture of the chemical structure of cartilage. Its water content is high, approximately 87 per cent. This is well shown by the tremendous collapse which occurs following the injection of crude papain; the epiphysial plate may decrease to one-third of its original width. The inorganic composition is approximately one-quarter of the dry weight. Hence, studies of incinerated sections reveal a prominent ash content in the nucleus, cytoplasm and matrix. Similar distribution is found if microradiographic techniques are applied.

The protein content may be studied by various procedures. DNA is found in the nucleus and there is prominent RNA in the cytoplasm. Small bluish masses interpreted to be mitochondria may be stained with Janus green. Similar aggregates are shown by the Nadi reagent in fresh tissue treated with ethyl carbamate, from which it is concluded that cytochrome oxidase activity is present. The tetrazolium procedure with various substrates indicates the presence of dehydrogenase activity. Alkaline phosphatase may be found in the cytoplasm of hypertrophic cells. Collagen has been identified chemically by the presence of hydroxyproline in isolated cartilage, free of bone and perichondrium, and doubtless is represented by the unbanded fibers seen with the electron microscope.

Of the carbohydrates, glycogen is present in large amounts in the young and mature hypertrophic cells. Alcian blue-staining material is present in the cytoplasm

of the cells and in the matrix as well; such structures are also metachromatic. Both stains probably indicate the presence of chondroitin sulphuric acid (CSA). Although the epiphysial cartilage contains about 0.8 per cent lipid, very little free fat is found in the cells by staining procedures. Neutral fat may appear in abnormal amounts, such as is found when cells are cultivated *in vitro* or in cartilage from rachitic animals.

Certain chemical reagents and enzymes have been utilized to gain a better understanding of the chemical composition of the cells and matrix. If fresh cartilage is treated by materials such as calcium chloride, hyaluronidase, papain, trypsin, etc., then fixed, sectioned and stained in various ways, some idea can be obtained as to the nature of the materials in the matrix. On the basis of such procedures it appears that one can remove the CSA with all of the above reagents, although only one of these, hyaluronidase, depolymerizes CSA. On the other hand, papain and trypsin, which do not act on collagen or CSA, must effect a loss of the latter material because of their digestion of the protein to which it is bound. Utilizing such procedures one gets a little clearer idea of the interrelations of chondroitin sulphuric acid, the protein to which it is complexed, and collagen.

The application of techniques such as these may now be utilized to obtain more information on the pathogenesis of certain experimentally produced disease states in cartilage. Today we are the recipients of "anatomical innovations" just as pathologists were a hundred years ago, when Virchow delivered his lectures on Cellular Pathology.

DISCUSSION

DOMINIC D. DZIEWIATKOWSKI: This has been a most delightful and revealing session to me, and I want to congratulate both speakers on their lucid presentations and on the experimental insight they have demonstrated in those presentations. As I sat listening, particularly to Dr. Follis, I was stimulated, and began to wonder what those pseudopods on the cartilage cells were there for. Is there any function that they have? Do they serve as channels for the excretion or secretion of material which the cell manufactures? I imagine at this time we can do nothing but speculate on that, but I hope sometime in the future someone will tell us what purpose they do serve.

The second thing that came to my mind is that perhaps now we should stop emphasizing the importance of collagen, although to some extent undoubtedly it is important. Instead we should place more emphasis on the mucoproteins and proteins associated with chondroitin sulfate, to which both speakers referred. These two points I think are the most important that were brought out, in addition to the indications that we have certain techniques available by which we may now be able to answer the question as to what role chondroitin sulfate, or the chondroitin sulfate associated with protein, has in the processes of calcification and ossification. In addition to this, I can add very little, and this "little" concerns the background against which both of the presentations of this evening may be viewed. We have made some observations on the possible use of S^{35} as an index of the metabolism of chondroitin sulfate. If one gives S^{35} to an animal, most of it, as Dr. Follis has indicated, is excreted rapidly in the urine and feces; within 24 hours about 80 per cent is eliminated. The remainder is fixed in the tissues which contain polysaccharides.

Within the epiphysial cartilage of a young rat S^{35} accumulates rapidly. Within 24 hours the peak is reached, and thereafter the S^{35} disappears slowly. If one examines these cartilages by autoradiography to determine the localization of the S^{35} within the bones as a whole, the material having been fixed in acidic formalin, the results of autoradiography appear as here (demonstrating on screen). You can see that there is an uneven distribution of S^{35} within the epiphysis. The highest concentration is within the epiphysial plate. The amount of the label increases within these regions rather rapidly; within 24 hours it reaches its peak and then begins to disappear, and to an obvious extent from regions of secondary ossification. In some preparations, that is, in the

humeri from the same animals, if fixed in formalin, saturated with barium hydroxide, the label is removed from the epiphysis, but one does see S^{35} throughout the rest of the bone, the diaphysis, and what is most interesting, as the label disappears from the epiphysial plate region, there is an accumulation of more and more label in the metaphysis. Stained preparations of sections of bones shown previously appear as these do. This is formalin-fixed material stained with toluidin blue.

If one looks at autoradiograms prepared in another fashion, by spreading a thin emulsion over sections, and this emulsion is retained during the subsequent processing, one can see that shortly after the administration of the labeled sulfate most of the label is concentrated within the cell, very little of it is out in the matrix. At eight hours the matrix begins to show more of the label, at 24 hours there is a rather even distribution between cells and matrix, and at 48 hours some of the cells begin to show up nearly free of the isotope. This cellular participation in the synthesis of chondroitin sulfate can be seen better in this preparation, again an autoradiogram, this time of a section of cartilage which first had been incubated *in vitro* in a solution of salts which has S^{35} in it. One can see that the autoradiographic reaction is intracellular, since in this preparation of calf-rib cartilage the chondrocytes are fewer and at greater distances from each other. For comparison of the cellular distribution in such a section, here is a stained preparation.

If you will recall the autoradiograms of the humeri, shown at the beginning of this discussion, there was a progressive loss of S^{35} from the cartilage plate and an accumulation in the trabeculae of the metaphysis. We were interested in trying to see what the nature of the material in the metaphysis might be, and we therefore undertook some chemical analyses. The results of these experiments as shown here indicate that in the epiphyses, diaphyses or metaphyses very little S^{35} labeled sulfate is retained as inorganic sulfate. By isolation of the remainder of the labeled materials from these tissues it was possible to demonstrate that the label in the trabeculae of the metaphysis was associated with chondroitin sulfate, or material like it.

KARL MEYER: I also want to preface my remarks with the statement that we were privileged tonight to hear two very provocative and stimulating papers. The problems dealt with by the speakers, of course, are a challenge to the biochemist as well as to the pathologist. I consider it rather unfortunate that we know so little about the biochemistry of epiphysial cartilage, while hyaline cartilage of trachea and of nasal septum has been studied quite extensively. What holds true for trachea may not be true for epiphysial cartilage. For example, the water content of tracheal cartilage is quite low, in the order of 60 per cent, while Dr. Follis finds in the epiphysial cartilage 87 per cent water. In connective tissue, we believe high water content to be associated with nonsulfated mucopolysaccharides.

We have learned from our investigations on the mucopolysaccharides of connective tissues from different sources that the types of mucopolysaccharides vary in different tissues, both in the types and in their distribution. From the studies of Schubert and his associates, as well as from those of other laboratories, we know that the sulfated mucopolysaccharides occur as complexes of a protein or proteins of still unknown type. It is not known whether or not these proteins are specific for various types of connective tissues or whether they differ with the various types of sulfated mucopolysaccharides. At this stage of our knowledge, it is not possible to give a chemical interpretation of the histological or histochemical data we heard tonight. In our studies on the rather mixed tissue of the long bones of the calf, including the epiphyses, we isolated mucopolysaccharides which are not found in resting hyaline cartilage. Thus we isolated from calf bone in addition to chondroitin sulfate A and C, the only identified mucopolysaccharides of hyaline cartilage, hyaluronic acid, keratosulfate and a large and probably nonhomogeneous chondroitin sulfate fraction, characterized by its incomplete sulfation. Dr. Follis showed in his slides the strong PAS-positive material. I

wonder what the nature of this material is. The isolated acid mucopolysaccharides do not react, or only very slowly, with periodic acid. Another problem arises from the action of papain and testicular hyaluronidase on the tissue slices. The molecular weight of chondroitin sulfate A and C is approximately 45,000 as found especially by Mathews in Chicago. We have to assume that the somewhat rigid rods or coils of this molecule in the native protein complex are bridged by protein. The protein complexes in turn by secondary valences are linked to the collagen fibers forming the cementing substances which bind the fibers into collagen bundles. When papain acts on the protein complexes, we assume that it breaks some peptide bonds of the protein. The question then arises whether the peptides thus formed are still linked to chondroitin sulfate. Conversely when hyaluronidase acts on the complexes, we would expect that the protein is left still attached to an oligosaccharide.

THEODORE TSALTAS: I would like to make some comments about the work Dr. McCluskey mentioned tonight. We have been doing some chemical determinations, on experimental animals similar to the ones Dr. McCluskey mentioned. I think some of the determinations were performed on the same animals. As Dr. McCluskey mentioned, many of our experiments were also performed 24 hours after the injection of papain. We had animals as controls and animals sacrificed 24 hours after the injection of papain. We noted a decrease of mucochondroitin sulfate of about 40 per cent, and also the S^{35} content of such mucochondroitin sulfate was 40 per cent less. Dr. McCluskey showed some pictures where there was some basophilic material in the tissues surrounding the cartilage, and the question was asked, where did that material go? We have some evidence that these sulfur-containing compounds are circulating in the blood in various forms. We isolated materials of various molecular weights, organic material that cannot be precipitated with trichloroacetic acid, and organic material that can be precipitated with trichloroacetic acid, as well as inorganic S containing materials. All these three classes showed increased concentration in the blood stream after the injection of papain, and some S^{35} was excreted in the urine. The increase was about 50 per cent over the controls for a 24 hour period. These experiments were performed 24 hours after the injection of papain. We did a series of experiments in which the animals were sacrificed every 4 hours up to 72 hours after the injection of papain. The maximum effect of the injection of papain occurred 12 hours after the injection. It lasted at about the same level for another 12 hours, that is, 24 hours from the injection of papain. The difference here is that Dr. McCluskey has observed that the changes persist for several days, while from our chemical determinations we found that the amount of chondroitin sulfate was almost at the normal level at 60 hours after the injection of papain. Also the amount of S^{35} in the blood stream reached the maximum 12 hours after the injection of papain and started decreasing steadily until it reached a low point at about 60 hours after the injection of papain. There is a point at which we do not understand definitely what is happening, that is, the S^{35} returns to the cartilage in some form that we do not know, but when we measure the S^{35} activity in cartilage 60 hours after the injection of papain, there is definitely S^{35} in larger amounts than were there 12 to 24 hours after the injection of papain.

A very interesting change was found in the $\frac{\text{nitrogen}}{\text{hexosamine}}$ ratio of the isolated mucochondroitin sulfate. This ratio changed by virtue of increased N_2 and decreased hexosamine. This change reached a maximum 12 to 24 hours after the injection of papain and returned to normal levels approximately 60 hours from the time of injection. This change in ratio can possibly give an indication of what happens in the molecule of mucochondroitin sulfate. It seems to us that papain breaks the molecule in such a fashion as to liberate hexosamine in larger amounts than peptides resulting in the ratio changes and the difference between control and treated animals. I cannot say that we are 100 per cent sure of this, be-

cause our material has to be purified, and the small remnants of cartilage have to be removed by hydrolysis before we have 100 per cent evidence, but the method by which we extracted the chondroitin sulfate allows us to be sure that something like this might have been happening.

Also I should like to add a word about the possibility of having edema in the tissue surrounding the cartilage: from our experience the ratio of the wet and dried cartilage in treated and control animals, as far as the weight was concerned, was the same: 3 to 1. As the wet cartilage and the papain-treated cartilage had exactly the same weight ratio, there is no possibility that the accumulation of water might have changed the properties.

I should like to make a comment about the staining methods mentioned by Dr. Follis. In some experiments that we did over the last two months we have been able to fractionate Sudan II (or Oil Red "O"), and we have found four compounds. One component stained fatty acids; two other components stained lipids, while a fourth stained proteins, so I do not know what those materials presented tonight might consist of, when stained with the mixture of these four components.

ALBERT SOBEL: I should like to compliment the contributors to this session for being most stimulating. I should like to ask Dr. Follis two questions: First, did he find any particular difference between preosseous and ossifying cartilage matrix? Second, does he think that alcian blue and toluidine blue stain two different substances, or different modifications of the same substance?

I should like to ask Dr. Dziewiatkowski: Is it possible that barium interferes with the metachromatic reaction of chondroitin sulfate?

DOMINIC D. DZIEWIATKOWSKI: It is possible that barium ions could interfere and mask the metachromatic reaction of chondroitin sulfate. In our preparations of cartilage fixed in formalin saturated with barium hydroxide, however, we feel that chondroitin sulfate is no longer present. This is supported by the fact that during the process of fixation S^{35} is lost from the cartilage.

KARL MEYER: I should like to ask whether changes have been observed in tissues other than cartilage. I believe Dr. McCluskey said there were no changes in other tissues following papain injection. I wonder whether studies have been carried out on the binding of papain by tissues other than cartilage. I have specifically in mind aorta in which the chief mucopolysaccharide fraction is chondroitin sulfate A, apparently identical with that of cartilage.

MARTIN BURGER: It was gratifying to learn that Dr. Follis observed that the collagen in the matrix was not banded. There seems to be some controversy about the calcifiability of banded and unbanded collagen. The M. I. T. group in Boston[7] believe that only 640 Å banded fibrils will calcify *in vitro*. We in our laboratory have repeatedly obtained *in vitro* calcification of chondroitin sulfate precipitated acid soluble collagen and this material is not banded, according to Fitton Jackson and Randall[8]. I just wanted to point this out for further comment by Dr. Follis.

ROBERT T. McCLUSKEY: In answer to Dr. Meyer's question, we have no evidence that papain has any effect on the mucopolysaccharides of the aorta or of other connective tissue and up to the present time we have no evidence that papain is concentrated in any site except cartilage. We may be able to find that out by using *in vitro* techniques comparable to that of the isolated rabbit's ear.

THEODORE TSALTAS: Dr. McCluskey had given me a couple of corneas and aortas of his animals, and I had taken some of my own, and when we did determinations on these materials (there were very few animals, I do not claim this will be of any particular value, except as an initial investigation) we found a decrease in the amount of S^{35} in the cornea and aorta of the treated rabbits. The decrease of S^{35} was not as striking as in the cartilage, but that

is as far as we went; I have not done any further work on it.

R. H. FOLLIS, JR.: To answer Dr. Sobel, the answer is, as far as calcification goes, "no". That is, there is nothing one sees in the calcifying area that is different from any others.

I do not know what the collagen fibers, whether banded or not, have to do with the calcification mechanism. The fact that fibers in cartilage do not show periodicity would make the hypothesis of Glimcher and associates open to some question. What the calcification mechanism is continues to be a mystery, to me at least.

As far as the question that Dr. Sobel asked about alcian blue and toluidine blue, this has been somewhat hard to interpret. The alcian blue apparently stains chondroitin sulfate; toluidine blue does also, but apparently stains something else; at least, that is our interpretation. I might add that we have applied a lot of other things to cartilage, which I did not show you; diastase and pancreatin work in the expected fashion.

I wish Dr. Meyer had drawn the little picture tonight which he drew for me yesterday and which explains this very lucidly. It made things very understandable and I think helps explain the results one gets *in vivo* and *in vitro*. The CSA molecules, which are long structures, may be bound together end to end by molecules of protein. These then may in some fashion be bound to collagen molecules, which are claimed to be 3000 Å in length and 12 or 13 Å in diameter. If one applies trypsin or papain, that is, proteolytic enzymes, of which the former does not act on collagen, and papain, which as far as I know, does not either, we break up the protein and doubtless liberate whole molecules of chondroitin sulfate, perhaps bound to the degradation product of this protein. If you incubate the tissue with hyaluronidase, you break up the CSA molecule itself and may at the same time liberate protein, perhaps that which was attached to the collagen molecules, so that you get rid of the alcian blue staining and some of the metachromasia. The concept of Dr. Meyer was extremely helpful to me and perhaps may be to you.

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